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Spontaneous Reversion of Human Immunodeficiency Virus Type 1 Neutralization-Resistant Variant HXB2thr582: In Vitro Selection against Cytopathicity Highlights gp120-gp41 Interactive Regions

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Spontaneous revertants of the immune-selected variant HXB2thr582, which resists neutralization by certain conformationally dependent antibodies specific for the CD4-binding site on gp120 (such as F105), appeared after long-term culture in the absence of immune-selecting serum. Molecular analysis showed some of the viruses in the revertant stock contained a simple back mutation, whereas others retained the Thr-582 codon but contained a substitution of serine for phenylalanine in gp41 at position 673. Neutralization sensitivity to the selecting serum and to F105 of infectious clones containing either the back mutation or the compensatory mutation, HXB2thr582ser673, was confirmed. HXB2thr582-infected cells have a greater propensity for syncytium formation and single cell killing than do either the parental HXB2 or the revertant HXB2thr582ser673. This suggests that the revertant arose by selection in vitro for a less cytopathic virus. Our results link three envelope regions shown to influence virus-cell fusion as well as neutralization by antibody: the CD4-binding region, the leucine zipper domain, and a region hidden to antipeptide antibodies upon envelope oligomerization. Taken together they illustrate the functional importance of the gp120-gp41 interaction and emphasize the impact of the interplay between envelope regions on overall conformation and function and on recognition by neutralizing antibodies.

Human immunodeficiency virus type 1 (HIV-1) exhibits extensive genetic heterogeneity, particularly in the envelope gene (23, 63). The resulting amino acid sequence variability in the viral surface glycoprotein, gp120, and transmembrane protein, gp41, provides the basis for the elicitation of type-specific antibodies. These in turn are able to exert immune-selective pressure which can lead to the propagation of variants in vitro and in vivo which have escaped immunologic control (1, 2, 31, 34, 46, 55). We previously described such an escape mutant derived by using a human high-titered neutralizing serum in an in vitro system to generate a neutralization-resistant variant of an infectious molecular clone of HIV-1, HXB2 (46). The molecular basis for the observed neutralization resistance was subsequently shown to be a point mutation in the *env* gene, resulting in the substitution of a threonine for an alanine residue at position 582 in the transmembrane protein (42). Further analysis of the variant, termed HXB2thr582, showed that the 582 region did not constitute a neutralizing epitope itself nor did it seem to be part of a noncontiguous neutralizing epitope. The results were best explained by a conformational change in the envelope protein, resulting from the alanine to threonine substitution and leading to alteration of a neutralizing epitope at another location (62). This hypothesis subsequently was confirmed by showing that HXB2thr582 is resistant to neutralizing antibodies which recognize a conformational epitope overlapping the CD4-binding region on gp120 (26).

Following transfection of the molecular clone HXB2thr582 into H9 cells and long-term culturing in the absence of immune-selecting serum, we have repeatedly observed its reversion to neutralization sensitivity. Because the basis for its original selection was a conformational change, we expected that although restoration of the parental conformation and neutralizing phenotype could be due to a back mutation, it was equally likely to result from a compensating change elsewhere within the viral envelope. We therefore analyzed the revertant virus population to determine the molecular basis for its altered phenotype. In addition, in order to gain further insight concerning the functional importance of regions highlighted by our molecular studies and the nature of selection pressures, other than immunologic, leading to the propagation of particular variants, we investigated the biologic basis for reversion of HXB2thr582 to neutralization sensitivity.

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MATERIALS AND METHODS

Cells and viruses. The parental virus used in these studies was HXB2, an infectious molecular clone of HIV-1 (17). It is sensitive to neutralization by the original immune-selecting serum (46). As a neutralization-resistant variant we used the mutant HXB2thr582 created from HXB2 by site-directed mutagenesis (42). This mutant reproduces the base change and subsequent amino acid substitution of alanine to threonine at position 582 which was originally selected in vitro in the presence of the neutralizing serum. pHXB2gpt, HXB2thr582, and HXB2thr582ser673 viral DNAs were transfected by electroporation into COS-1 cells, and progeny virus was transmitted 24 h later by coculturing into H9 cells for propagation (39). The virus-producing H9 cells were cultured in the absence of immune-selecting serum in RPMI 1640 containing 10% fetal calf serum, 1 mM glutamine, and penicillin and streptomycin. Cell-free virus preparations from the parental and mutant cultures were assayed periodically for neutralization resistance or sensitivity to the original selecting serum.

Antibodies. The original selecting human serum, possessing high titer and broadly reactive neutralizing antibody, was obtained from a healthy HIV-seropositive individual and was used to determine neutralization sensitivity or resis-

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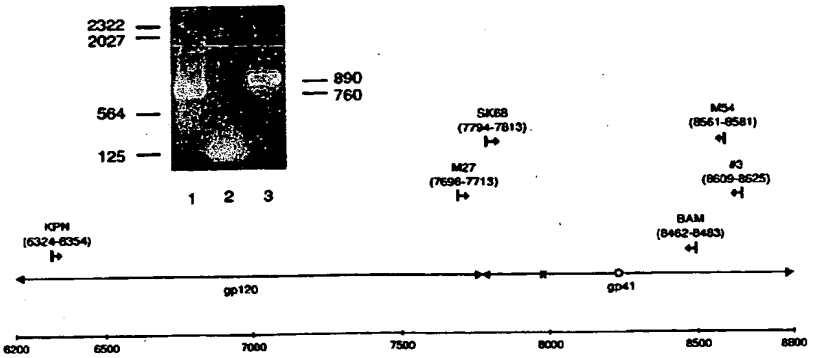

FIG. 1. Location of primers used for amplification of revertant envelope DNA. Amino acid position 582 is marked by an "×" and position 673 is marked by an "O." The insert shows the DNA products of the first (lane 3) and second (lane 1) PCR amplifications. The reaction of lane 2 contained no DNA. Migration of standard kilobase markers is indicated on the left, and the calculated size of DNA products is on the right.

tance of the revertant viral population and cloned variants. Normal human serum served as a control. The human monoclonal antibody F105 (40, 41), specific for a conformational determinant overlapping the CD4-binding region (53), was used as ascites fluid containing the antibody obtained from nude mice or as purified immunoglobulin G (IgG). Ascites fluid from mice inoculated with P3 × 63 hybridoma cells or purified human IgG was used as a control.

Immunologic assays. Neutralizing antibody assays were carried out as previously described (43, 44), using fresh cell-free supernatant of the virus-producing H9 cultures as the source of virus. The sensitivity or resistance to neutralization of the viral strains was assessed by using 1:10 dilutions of the original selecting serum or normal human serum as control and serial dilutions of virus. Determination of neutralization sensitivity or resistance was made at viral dilutions at which the kinetics of infection of each of the viruses in the presence of control serum were similar. Viral infection of the target H9 cells was determined by expression of p24 as monitored in an indirect immune fluorescence assay (IFA) with an HIV-1 p24-specific monoclonal antibody.

Antibody binding to H9 cells infected with HXB2, HXB2thr582, and HXB2thr582ser673 was assessed by FACScan analysis as previously described (45) by using a pooled human serum, purified IgG of the F105 monoclonal antibody, and human IgG as control.

Assessment of virologic properties. Production of infectious virus and viral proteins was determined by measuring p24, reverse transcriptase (RT), and gp120 levels of expression in supernatant media of infected cells. Levels of p24 and gp120 were quantitated with commercially available antigen capture kits (Coulter, Hialeah, Fla., and American Bio-Technologies, Cambridge, Mass., respectively). Virus particles were precipitated with polyethylene glycol from cell-free supernatant as previously described (39). RT activity was measured by using oligo(dT_{12-18})-poly(A) as template in the presence of Mg^{2+} as previously described (38). The percentage of infected cells was determined by IFA on cells fixed for 10 min at room temperature in 50% methanol–50% acetone, using an HIV-1 p24-specific monoclonal antibody.

Syncytium formation by virus-infected cells was determined with uninfected CEM-SS, SupT-1, and HUT 78 cells as targets. Twenty-five-microliter dilutions of infected cells, ranging from 1×10^5 to 5×10^5/ml were mixed with 25 μl of CEM-SS cells (10^6/ml) in a total volume of 100 μl in half-well, flat-bottomed, 96-well microtiter plates. Syncytia were counted after overnight incubation at 37°C. The number of syncytia per 10^3 p24-expressing infected cells (determined by IFA) was calculated from the results of triplicate determinations.

Single-cell killing was assessed by infecting H9 cells with equivalent amounts of either HXB2, HXB2thr582, or HXB2thr582ser673 and culturing for 3 weeks. Total cell count and viability by trypan blue staining and percentage of cells expressing viral p24 by IFA were determined every 2 days, at which time the cultures were adjusted to 10^6 viable cells per ml in fresh medium.

Amplification of DNA for direct sequencing. Cells (2×10^7) producing the uncloned revertant virus were digested for 18 h at 50°C with 100 μg of proteinase K per ml in TNE (0.01 M Tris-HCl buffer [pH 9.0], 0.1 M NaCl, and 1 mM EDTA) containing 0.5% sodium dodecyl sulfate. Genomic DNA was extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1) equilibrated to pH 9.0 and twice with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with ethanol, washed twice with 80% ethanol, and resuspended in water.

One microgram of genomic DNA was used as the template for amplification by PCR. Mixtures contained 0.5 μg of each primer, 3.5 U of *Taq* DNA polymerase, and 5% glycerol in addition to components specified by the Perkin-Elmer Cetus (Norwalk, Conn.) PCR kit instructions. Thirty amplification cycles were carried out, with 45 s at 94°C for denaturation, 1.5 min at 50°C for annealing, and 2 min at 72°C for extension. The primers used (M27 and no. 3) are specified in Fig. 1.

DNA from 15 μl of the first amplification was separated electrophoretically on a 1% agarose gel. The appropriately sized DNA product (890 bp) was excised from the gel and freeze fractured by freezing overnight at 20°C and subsequently thawing at 37°C for 5 min. DNA was eluted following maceration of the agarose by suspending it in 400 μl of TE (0.01 M Tris-HCl buffer [pH 7.4], 1 mM EDTA), transferring to a Millipore (Bedford, Mass.) ultrafree MC 0.45-μm-pore-size filter unit, and centrifuging at 12,000 rpm for 30 min. DNA in the flow through was concentrated by ethanol precipitation and resuspended in 10 μl of sterile water.

One microliter of the purified first amplification product was subjected to a second round of amplification under the same PCR conditions as outlined above (without 5% glycerol) by using 0.5 μg of nested sense and antisense primers (SK68 and M54) (Fig. 1). Fifteen microliters of the second amplification product was electrophoretically separated and purified as described above. The double-stranded DNA product, concentrated by ethanol precipitation, was dissolved directly in appropriate buffer for sequencing by one of two methods.

The ^{32}P-end-labeled primer extension method was used in association with an altered Sequenase (version 2.0) protocol of the U.S. Biochemical Corp. (Cleveland, Ohio) (32). The DNA product was dissolved in 12 μl of ^{32}P-end-labeled primer (2 ng/μl) to which 2 μl of Sequenase reaction buffer was added. The DNA product and ^{32}P-end-labeled primer mixture were denatured in boiling water for 2 min and then annealed by snap-cooling for 10 min in an ice-water bath. Components of the Sequenase protocol were added, and the procedure was carried out as described by the manufacturer. No labeling mix or ^{35}S-labeled dATP was required, because the primer was already labeled. Alternatively, the double-stranded cycling procedure of Applied Biosystems Inc. (Columbia, Md.) and Promega (Madison, Wis.) was followed by using the dyedeoxy terminator *Taq* sequencing kit and analyzed on a model 373A DNA sequencer according to the directions of the manufacturer. In either case, the sequencing reaction mixtures were electrophoresed on 8% sequencing gels, followed by autoradiography or laser-fluorescent scanning as appropriate.

Clonal analysis of revertant DNA. A single round of PCR amplification of revertant genomic DNA with a set of primers (KPN and BAM) (Fig. 1) encompassing most of the envelope was carried out as described above. The components and the cycle parameters of the PCR were identical to the procedure described above. The DNA product was purified as described above, concentrated by precipitation in ethanol, and dissolved directly in appropriate buffer for digestion by the restriction enzymes *Kpn*I and *Bam*HI (Boehringer Mannheim, Indianapolis, Ind.). One microgram of the Bluescript cloning vector (Stratagene, La Jolla, Calif.) was similarly digested, and the cut DNA product and cloning vector were electrophoretically separated and purified as described above. The DNA products from both were dissolved and combined in a total of 20 μl, including 2 μl of ligation buffer and 1 U of T4 DNA ligase (Boehringer Mannheim). The ligation mix was incubated for 16 h at 10°C. Two microliters of ligation mix was added to 100 μl of thawed HB101-Max-competent cells according to the protocol of GIBCO-BRL (Gaithersburg, Md.) and incubated on wet ice for 1 h. The cells and ligation mix were incubated at 42°C for 45 s and placed on ice for 2 min. Nine-hundred microliters of SOC medium (GIBCO-BRL) was added to the competent cell mix, and the cells were grown on a shaking water bath at 37°C for 1 h. The transformed cells were grown on agar plates containing ampicillin (50 μg/ml) for 16 h at 37°C. The colonies were screened by minipreprestriction digest analyses.

Positive minipreps were expanded, and plasmids (cloning vector plus ligated PCR DNA) were isolated and sequenced according to the Sequenase double-stranded DNA protocol (U.S. Biochemical Corp.).

Construction of biologically active clones. The two-step scheme used to construct infectious molecularly cloned viruses containing the original Thr-582 and

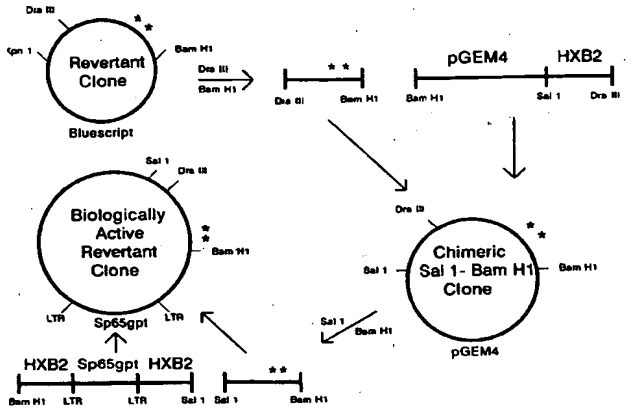

FIG. 2. Construction of proviral clone of revertant virus. A pGEM4 plasmid containing the *Sall-Bam*HI portion of HXB2 was digested with *Dra*III and *Bam*HI, purified, and used as a vector into which the *Dra*III-*Bam*HI fragment from either HXB2thr582ser673 or HXB2arg521thr582 was inserted. The mutations coding for Thr-582 and Ser-673 are illustrated (*). From these chimeric *Sall-Bam*HI clones, the *Sall-Bam*HI fragment containing either HXB2thr 582ser673- or HXB2arg521thr582-derived sequence from *Dra*III to *Bam*HI was purified and inserted into the large *Sall-Bam*HI fragment of HXB2 to generate the biologically active revertant clone. These procedures are outlined schematically.

compensatory mutations is illustrated in Fig. 2. One microgram of the intermediate vector DNA pGEM4 (Promega), containing the *Sal*1-*Bam*HI fragment from the HXB2 *env* gene, was digested with *Dra*III (New England Biolabs, Beverly, Mass.) and *Bam*HI. The *Dra*III-*Bam*HI fragment of the revertant *env*, containing the two mutations, was then ligated into the cut intermediate vector. Positive plasmids were isolated by ampicillin selection in HB101-Max-competent cells as described above and selected further by miniprep analyses.

One microgram of the intermediate construct plasmid and pHXB2/RIP7 (13), an infectious proviral clone of HXB2, was digested with *Sal*I and *Bam*HI (Boehringer Mannheim), electrophoretically separated, and purified, the latter with the Gene Clean protocol (BI0 101, La Jolla, Calif.). The insert and vector were ligated, and positive clones were isolated by ampicillin selection and miniprep restriction analyses as described above. In order to verify that only the two point mutations were present, both strands of the envelope gene of the infectious molecular clone designated HXB2thr582ser673 were sequenced as described above for the revertant clones after PCR amplification of DNA by a series of primer pairs, each spanning 150 to 200 bp of the gene.

RESULTS

Reversion of the HXB2thr582 mutant to neutralization sensitivity. Following transfection of HXB2thr582 into permissive cells, we have repeatedly observed that after prolonged periods of culture in the absence of selecting serum, the HXB2thr582 variant loses its property of neutralization resistance to the original selecting serum. In order to document this phenomenon and to study the molecular and biologic bases for the reversion, HXB2thr582 and HXB2 DNAs were transfected into COS cells and progeny virus was transmitted to H9 cells. The cultures were maintained without immune-selecting serum, and the neutralization phenotypes of cell-free supernatant viruses were assessed periodically. HXB2thr582 maintained its neutralization-resistant phenotype in the absence of selecting serum for at least 6 weeks of culture as illustrated in Fig. 3. With continued culture in the absence of selecting serum, the virus produced gradually became more neutralization sensitive. By 3 months of culture, transmission of cell-free virus from the HXB2thr582 supernatant to H9 cells in the presence of the original immune-selecting serum was significantly retarded. In contrast, the kinetics of infection of H9 cells in the presence of normal human serum by either the control

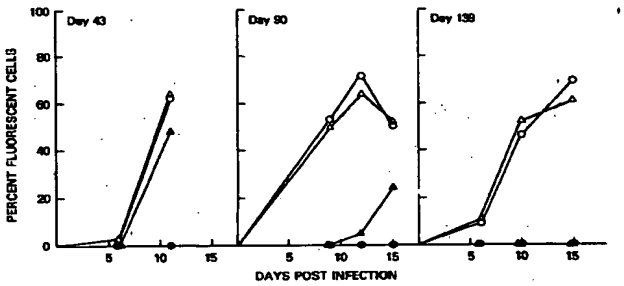

FIG. 3. Loss of neutralization resistance of the HXB2thr582 variant to the original selecting serum. HXB2thr582 and HXB2 infectious DNAs were transfected into COS-1 cells, and the virus produced was transmitted to H9 cells by coculturing. The cultures were maintained in the absence of immune-selecting serum, and the neutralization phenotypes of cell-free virus preparations were assessed on the days indicated. The kinetics of secondary transmission of HXB2thr582 (Δ) and the parental virus HXB2 (O) to H9 target cells in the presence of normal human control serum (open symbols) or the original immune selecting serum (closed symbols) is illustrated.

HXB2 virus or the passaged HXB2thr582 supernatant were identical, indicating that the lessened rate of infection by HXB2thr582 in the presence of selecting serum did not merely reflect quantitatively less virus in the cell-free supernatant. By 4.5 months, virus produced in the HXB2thr582 culture was completely sensitive to neutralization by the immune-selecting serum, indicating total reversion to the neutralization phenotype of the parental HXB2 virus (Fig. 3).

Direct sequencing of the revertant DNA. In order to determine the molecular basis for the reversion to parental phenotype, genomic DNA extracted from H9 cells producing the revertant virus after 5 months of continuous culture was amplified by PCR. As illustrated in Fig. 1 (inset) a second amplification with nested primers resulted in a significantly greater yield of DNA which facilitated direct sequencing. The secondary DNA product, composed of approximately 760 bp encompassing the 582 region of the envelope was sequenced directly as described in Materials and Methods. The resulting consensus sequence revealed that a simple back mutation consisting of a single nucleotide change of A to G had occurred, resulting in an alteration of the threonine codon to alanine at amino acid position 582 (Fig. 4). However, inspection of the sequencing gel suggested that the revertant virus population was mixed, as a proportion of the amplified DNA retained the A (Fig. 5). An approximation of the relative amounts of revertant DNA containing either the A or the back-mutated G was carried out by quantitation with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). As illustrated in Fig. 5, the back mutation was approximately three times more abundant than the parental genotype. The mixed genotype observed in the amplified revertant DNA suggested that a compensatory mutation elsewhere in the *env* gene might have occurred in some of the virus population, also leading to neutralization sensitivity.

Clonal analysis of revertant DNA. To further investigate the mixed revertant population, a 2,159-bp region encompassing most of the envelope gene including the portion encoding the 582 region (Fig. 1) was amplified by PCR and introduced into a cloning vector. Eight clones were obtained and sequenced as described in Materials and Methods. Of the eight clones, four contained the A to G back mutation and four retained the A which would preserve the threonine characteristic of the variant at position 582. Of the latter four clones, one contained a stop codon and one lacked the *Bam*HI site and were not

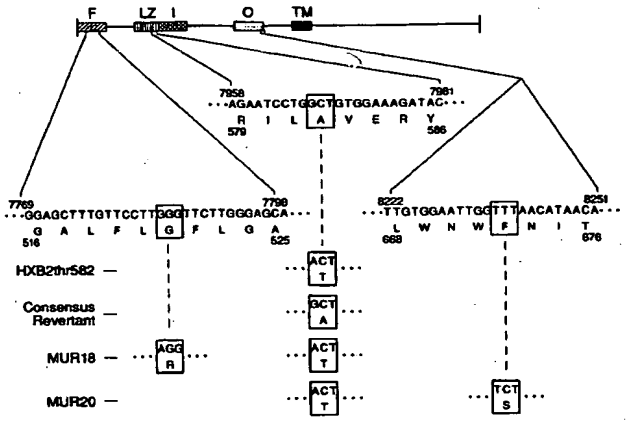

FIG. 4. Molecular analysis of the HXB2thr582 revertant. The parental HXB2 envelope protein is represented schematically at the top, with the fusion peptide (F), leucine zipper region (LZ), immunodominant region (I), a site hidden upon envelope oligomerization (O), and transmembrane region (TM) localized according to the systems in references 8, 10, 21, and 35. Base changes and deduced amino acid alterations occurring in the original variant HXB2thr582, the consensus revertant obtained by direct sequencing of PCR-amplified DNA, and two cloned envelopes, MUR18 and MUR20, are illustrated.

examined further. The third clone, designated MUR18, exhibited a G to A mutation which would result in a glycine to arginine substitution at position 521 in the envelope protein (Fig. 4). The fourth clone, MUR20, possessed a change of T to C, resulting in alteration of a phenylalanine codon to a serine codon at amino acid position 673 (Fig. 4).

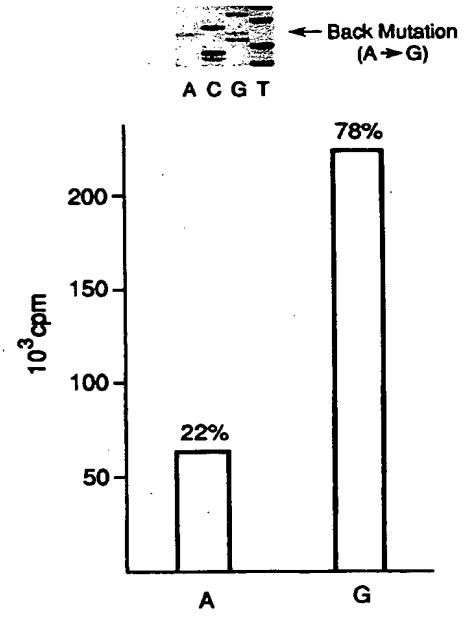

FIG. 5. Consensus sequence of revertant virus and estimation of the extent of the mixed population. Three separate PCR amplifications of DNA from the revertant virus were sequenced. The inset shows a portion of the first sequencing gel in which a mixture of adenine and guanine is present at a single site. The bar graph represents the results of a second sequencing of this region; the relative proportions of adenine and guanine were determined by phosphorimaging analysis. A third sequencing also revealed a mixed population (not shown).

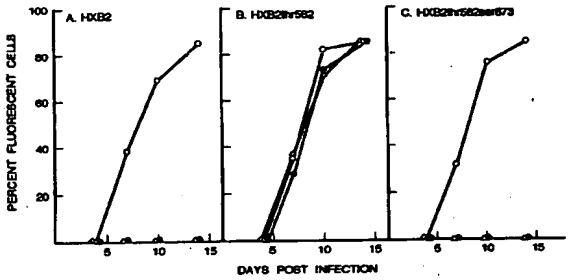

FIG. 6. Compensatory mutation at position 673 restores neutralization sensitivity. The kinetics of infection of H9 cells by the viruses shown was determined in the presence of a normal human control serum (○), the original human serum used to select the HXB2thr582 escape mutant (●), and monoclonal antibody F105 (△). Virus expression was monitored by immune fluorescence using a monoclonal antibody specific for p24 as described in Materials and Methods.

In order to determine if the secondary mutations in MUR18 and MUR20 would compensate for the original alanine to threonine substitution, the cloned envelope genes were substituted into the infectious molecular clone HXB2 as described in Materials and Methods and Fig. 2. Following transfection of the clone containing the MUR18 envelope into H9 cells, no progeny virus was produced and this clone was not examined further. The clone containing the MUR20 envelope was infectious. Sequencing of the complete MUR20 envelope gene confirmed that only the two codon changes relative to HXB2 were present (not shown), and the clone was redesignated HXB2thr582ser673. Neutralization analysis confirmed that the compensatory mutation at position 673 restored neutralization sensitivity to both the original immune-selecting serum and to monoclonal antibody F105 (Fig. 6). The latter, a human monoclonal antibody specific for a conformational epitope overlapping the CD4-binding region of gp120 (40, 41, 53), was previously shown to be highly discriminatory for HXB2 versus HXB2thr582, neutralizing the former but not the latter (26).

Characterization of H9 cells infected with HXB2, HXB2thr582, and HXB2thr582ser673. The ability of the F105 monoclonal antibody to bind to cells infected with HXB2, HXB2thr582, or HXB2thr582ser673 was assessed by FACScan analysis. As shown in Table 1, virus expression by each of the three infected cell types was similar as judged by staining with a pooled human serum seropositive for HIV. However, whereas ap-

TABLE 1. Binding of monoclonal antibody F105 to H9 cells infected with parental, neutralization-resistant variant, and neutralization-sensitive revertant cloned viruses[a]

Virus	% PHS[b]-positive cells	Binding of F105	
		% Positive cells[c]	Relative affinity (μg/ml)[d]
HXB2	90	58	3.0
HXB2thr582	85	25	3.2
HXB2thr582ser673	87	35	3.2

[a] Values represent the mean of three experiments.
[b] PHS, pool of three human sera with broad HIV-1 envelope reactivity.
[c] Normalized to percent positive cells stained with PHS.
[d] Defined as the antibody concentration at which a 50% reduction in mean fluorescent intensity of cells stained by a dilution series of F105 antibody was observed.

TABLE 2. Virological characteristics of parental, neutralization-resistant variant, and neutralization-sensitive revertant cloned viruses[a]

Virus	Viral protein expression in supernatant media			Syncytium formation[b] in indicated target cell		
	RT activity	p24	gp120	SupT-1	HUT 78	CEM-SS
HXB2	1.00	1.00	1.00	1.00	1.00	1.00
HXB2thr582	1.08	0.81	0.88	2.48	2.18	2.10
HXB2thr582ser673	0.86	0.89	ND[c]	1.40	1.20	1.25

[a] Results (average of two to four experiments) are expressed relative to the value for the HXB2 parental virus.

[b] The number of infected cells was first calculated based on the percentage of cells in the culture expressing p24 by IFA. Syncytium formation per 10^3 p24-positive infected cells was subsequently calculated and normalized to HXB2 values.

[c] ND, not determined.

proximately 60% of cells infected with HXB2 were able to bind monoclonal antibody F105, only 25% of cells infected with the neutralization-resistant variant were stained by the antibody. The compensatory mutation in the revertant HXB2thr582ser673 partially restored recognition of the F105 epitope on the surfaces of cells expressing the viral envelope to the level seen with HXB2-infected cells. The relative binding affinity of F105 for the viral envelope expressed on the cell surface as determined by FACScan analysis was similar for all three infected cell types (Table 1).

In order to determine the nature of the selection pressure for reversion of HXB2thr582 to the parental phenotype, the levels of expression of viral proteins and the propensity of infected cells to form syncytia were determined for H9 cells infected with HXB2, HXB2thr582, and HXB2thr582ser673. As shown in Table 2, supernatants from infected cultures were assessed for p24 and gp120 production and for RT activity. The levels of infectious virus production and protein expression by H9 cells infected with HXB2, HXB2thr582, or HXB2thr582 ser673 were equivalent, indicating that the revertant virus did not have a growth advantage. To further probe the basis for revertant selection, the ability of the virus-infected cells to form syncytia with $CD4^+$ target cells was investigated. Cells infected with HXB2thr582 consistently exhibited a greater number of syncytia upon coculture with either CEM-SS, SupT-1, or HUT 78 target cells (Table 2). Syncytium formation by HXB2thr582-infected cells was 2.10 to 2.48 times greater than that of HXB2-infected cells. HXB2thr582ser673-infected cells exhibited an intermediate level of syncytium formation.

The HXB2thr582ser673 revertant appeared in a culture of H9-infected cells. However, H9 cells as targets do not readily form syncytia. Therefore, we explored the possibility that increased cytopathic effect of the HXB2thr582 variant might also be expressed by greater single cell killing (4, 7, 47, 48). Figure 7 illustrates that this is the case. After infection with HXB2, HXB2thr582, or HXB2thr582ser673, H9 cells reached stable levels of productive infection within 6 days (Fig. 7A). Thereafter, virus expression by the infected cell populations ranged between 40 and 60% as measured by cells expressing the p24 core protein. Although the amounts of infecting virus were similar and the same level of productive infection was achieved by all three types of virus-infected cells, H9 cells infected with HXB2thr582 exhibited much more extensive single cell killing (Fig. 7B). As a result, HXB2- and HXB2thr582ser673-infected H9 cells consistently showed a higher total viable cell count beginning at approximately 1.5 weeks postinfection (Fig. 7C).

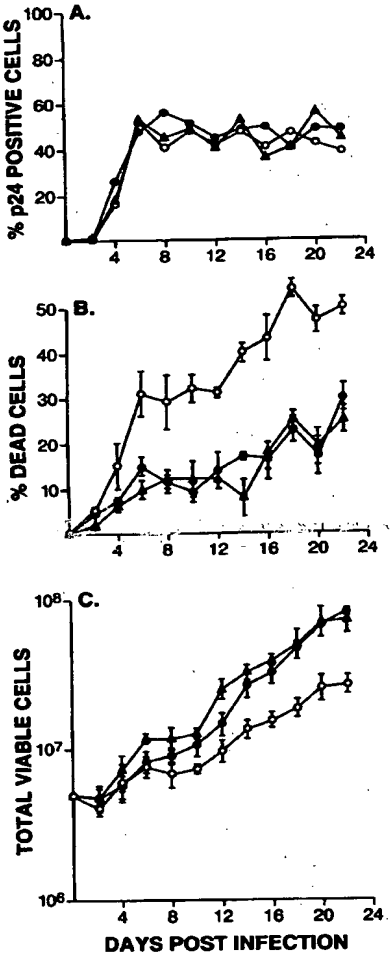

FIG. 7. Single cell killing by the parental, neutralization-resistant variant, and revertant viruses. (A) Kinetics of infection of H9 cells by the three viruses is shown. Virus infection was monitored by IFA for p24 expression. (B) Percentage of dead cells plus or minus the standard deviation. (C) Total number of viable cells plus or minus the standard deviation. Symbols: ●, HXB2; ○, HXB2thr582; ▲, HXB2thr582ser673. The experiment was repeated three times with similar results. A representative experiment is shown.

Although the experiment was terminated after 3 weeks of culture, it illustrates how the HXB2thr582 variant would be selected against in an in vitro system lacking any immune-selective pressure once the back and compensatory mutations were introduced into the viral population.

DISCUSSION

The HXB2thr582 neutralization-resistant variant exhibits an unstable phenotype in the absence of immune-selecting serum. Here we showed that the molecular basis for reversion to neutralization sensitivity is due, in part, to a back mutation at position 582. This result reflects the highly conserved nature of the 582 region and its functional importance. However, we also showed that reversion can be due to a compensatory change outside the 582 region. Compensatory changes can affect viral functions such as infectivity and cell tropism (33, 60, 61). But

HIV escape mutants perhaps best exemplify the effects of point mutations on conformational changes and their resultant influence on functions at distant sites. While some mutants escape because of amino acid alterations within a neutralizing epitope, either linear (31) or conformational (9), others, including the HXB2thr582 variant, escape via conformational changes affecting a site which is not part of the neutralizing epitope (26, 34, 56, 62). Here we have shown that the impaired recognition of the conformationally dependent CD4-binding site by neutralizing human polyclonal and monoclonal antibodies induced by the alanine to threonine substitution at amino acid position 582 can be functionally restored on cell-free virions by a compensating alteration of phenylalanine to serine at position 673.

Evidence of impaired recognition of the F105 epitope on cells expressing HXB2thr582 compared with HXB2 and an intermediate level of binding to cells expressing HXB2thr 582ser673 was obtained by FACScan analysis (Table 1). However, these results suggest that envelope conformation on the surfaces of virions must differ from that of envelope expressed on cell surfaces, because the decrease in F105 binding to HXB2thr582 does not seem sufficient to account for the remarkable ability of F105 to discriminate the parental from the neutralization-resistant virus in a functional assay. More than 81-fold more F105 is necessary to neutralize cell-free HXB2thr582 infection than that required to neutralize HXB2 infection (26). Moreover, the small increase in binding of F105 to cells expressing HXB2thr582ser673 compared with those expressing HXB2thr582 does not correlate with the complete restoration of functional F105 antibody activity observed with the compensatory mutation (Fig. 6). A similar lack of quantitative differences in binding to cell surface-expressed envelope by other neutralizing monoclonal antibodies which discriminate between HXB2 and HXB2thr582 virions was previously reported (26).

Virologic characterization of cells infected with the parental HXB2 virus, the original HXB2thr582 virus, and the HXB2thr 582ser673 virus suggested that the in vitro pressure responsible for reversion to the parental phenotype resulted from selection for a less cytopathic virus. Two distinct mechanisms lead to cytopathic effect in HIV-infected cells: syncytium formation and single cell killing (7). We showed that both mechanisms lead to increased cytopathicity of the HXB2thr582 variant (Table 2 and Fig. 7). The observed increase in syncytium formation by HXB2thr582 differs from the result of Thali et al., who reported that the Thr-582 mutation leads to a decreased ability to form syncytia (52). The different result may be due to differences in the systems studied. We used infectious molecularly cloned viruses to study natural infection of target cells, while Thali et al. use a complementation assay to study the effects of either wild-type or mutant envelope expression. In addition, we normalized the numbers of syncytia formed to the number of cells in each infected population actually expressing virus (Table 2). This was not done in our previous study (62), cited by Thali et al. (52), and thus cannot be compared with the results reported here.

In an in vitro tissue culture system in which HIV proviral DNA is integrated into a proportion of immortalized T cells, a highly cytopathic virus would be at a disadvantage. Cells containing such a provirus would more likely die, while a more benign viral type would be favored and tend to accumulate. The overall dynamics of the system and length of time for a revertant virus to be selected would depend on the proportion of cells initially infected and expressing virus and on the cell doubling time. A similar selection pressure probably does not operate in vivo, however. In the absence of immortalization, cells containing more benign proviruses would not tend to accumulate. In fact, selection for a more virulent virus might occur, as such a virus would tend to infect greater numbers of susceptible T cells. This speculation is supported by numerous reports illustrating the shift in viral characteristics from initially "slow-low," non-syncytium-inducing, macrophage-tropic strains early in infection to "rapid-high," syncytium-inducing, T-tropic strains later in disease progression (3, 15, 16, 51).

The nature of the molecular and biologic changes which occurred in the immune-selected variant and the more benign revertant illustrates important functional interactions between gp120 and gp41 envelope components and highlights several envelope domains believed to play a role in fusion events. The precise mechanism by which HIV fuses with cells is not known. The process is pH independent and thus involves direct fusion of the virus envelope with the cell membrane (49). Whereas gp41 by itself can induce fusion (37), the natural process involves the initial binding of gp120 to the CD4 receptor, followed by a conformational change which presumably uncovers the gp41 fusion domain, allowing fusion to occur. This mechanism may ensure the maintenance of viral tropism by preventing promiscuous fusion with cells lacking the CD4 receptor (37). In addition to the CD4-binding region, other gp120 regions influence virus-cell fusion, including V1/V2 (50) and V3 (5, 19, 22, 36, 54). The actual fusion peptide, however, is thought to be located at the amino terminus of gp41 (20). Mutations in this region have led to decreased viral fusion (5, 7, 14, 18, 28, 29). Other gp41 domains influence fusion events and viral function, however, including the leucine zipper, encompassing position 582 (6, 10); the transmembrane region and residues proximal to it (24); and the 673 region (6). This latter region has been shown to be inaccessible to specific antipeptide antibodies upon oligomerization of the viral envelope (35). However, oligomerization does not require the 673 region and can occur with only the first 129 amino-terminal residues of gp41 present (11). In fact, gp41 amino acids 68 to 129, encompassing the 582 region, are critical for envelope oligomerization (12). Further functional importance of the 582 and 673 regions has been demonstrated in recent studies in which synthetic peptides representative of both regions have displayed potent antiviral activities and inhibition of fusion (25, 57–59). These same two regions are postulated to interact and play a critical role in the fusion process and viral entry (30). The gp41 regions discussed are illustrated in Fig. 4.

We are intrigued that the mutations observed during the progression of the parental HXB2 virus through the neutralization-resistant variant to the spontaneous revertant involve the CD4-binding region in gp120 and the 582 and 673 regions in gp41, all believed to participate in fusion events. Furthermore, the changes observed in the fusion abilities of the three viruses lend support to the proposed functional roles of the three envelope regions. The initial variant selection resulted in the neutralization-resistant HXB2thr582 which had a greater propensity for forming syncytia (Table 2). The mutation in gp41 altered the CD4-binding region of the viral envelope such that recognition by conformationally dependent antibodies specific for the region was impaired (26). Binding to CD4 is a first step in the fusion process. Our results thus emphasize the functional importance of gp120-gp41 interactions in the initial events of viral infection.

The subsequent compensating change at position 673 which restored neutralization sensitivity to CD4-binding site antibodies and decreased syncytium formation to wild-type levels illustrates the interplay between the 582 and 673 envelope regions and indirectly supports previous findings implicating both regions in the fusion process. Helseth et al. (24) have

suggested that the fusion peptide and the transmembrane region interact with each other and/or with lipid membranes, resulting in fusion. The two α-helical regions between these domains, namely the 582 and 673 regions, must act to preserve conformation appropriate to allow fusion to occur. This argument is taken further by Matthews et al. who elegantly illustrate how the two regions may interact to form a "fusion attack complex" necessary for viral entry (30).

The nature of the amino acid substitutions selected in the variant and revertant viruses is of interest. The leucine zipper region does not allow many alterations without infectivity being impaired. Alterations at positions 585 and 586 have resulted in noninfectious viruses (62), and only conservative amino acid substitutions are permitted at position 573 in order for infectivity to be maintained (10). Position 582 can accommodate several amino acids and still retain infectivity (62). The biochemical basis for threonine-induced neutralization resistance by a conformational alteration involving the CD4-binding site is unknown, however. Because alanine, serine, and glycine residues at position 582 lead to neutralization-sensitive viruses (62), it has been suggested that a bulkier residue, such as the threonine, might also cause neutralization resistance (27). However, substitution of valine at position 582 of HXB2 produced a neutralization-sensitive virus (55a).

Cao et al. (6) have reported that, unlike our observations here, substitution of phenylalanine with a proline residue at position 673 led to increased syncytium-forming ability. In our case, substitution of the phenylalanine with serine produced a potential glycosylation site (NXT/S) (Fig. 4). Additional experiments are necessary to determine whether glycosylation at this site mediates the decrease in syncytium formation of the revertant and the effect of the serine substitution by itself on syncytium formation.

Overall, these studies highlight the conformational aspects of immune escape and demonstrate the clear interaction between gp41 and gp120, which is important for viral function. They illustrate that immune-selective pressure is not the only force contributing to viral variability. Finally, they show the interplay occurring between envelope domains which influence virus-cell fusion and emphasize the plasticity and adaptability of the viral envelope.

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